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PATENT- OG VAREMÆRKESTYRELSEN

Field of invention

The present invention concerns a method of producing growth hormone crystals in the presence of cations, the produced crystals and pharmaceutical preparations containing these crystals.

Background of the invention

The growth hormones (GH) from man and from the common domestic animals are proteins of approximately 191 amino acids, synthesized and secreted from the anterior lope of the pituitary. The growth hormone is a key hormone involved in the regulation of not only somatic growth, but also in the regulation of metabolism of proteins, carbohydrates and lipids.

During the past 40 years or more much attention has been devoted to the unravelling of the biochemical function of the growth hormones from various species. The reason for this interest in the molecular function of this polypeptide rests upon the commercial interests from both veterinarian and medical circles. The GH gene has now been cloned. Human growth hormone (hGH) and Met-hGH are currently beeing produced biosynthetically by the use of both bacteria and mammalian cell cultures.

After this production has started hGH is no longer limited for treatment of growth retardation but a larger and still increasing field of use has taken place, examples are wound healing and treatment of infertility.

Growth hormone is a protein with a relatively short halflife for which reason there is a need to find a way to prolong the release of GH into the blood stream. At the same time there is also a need to increase the stability of the preparations. An answer to this problems could be well defined and chemically stable GH crystals.

Although readily available in quantities sufficient for crystallization, GH has eluded succesfull crystallization. However, micro crystals, or amorphous material have been reported from a variety of sources: (Jones et al. 1987; Wilhelmi et al. 1948; Lewis et al., 1958; Adel-Mequid et al., 1986; 1987; Clarkson et al., 1989).

The hanging drop method is the most common method in use for this purpose. Apparently due to heterogenicity among growth hormone preparations the size and the shape of the crystals have been reported to vary significantly. The largest crystals have been reported by Jones et al. (1987). For their successfull experiments they used a mixture of polyethylene glycol 3500 and beta octyl glucoside at neutral pH. The size of the crystals were of a suitable size for X-Ray crystallography studies. Clarkson et al. (1989) reported that only the use of lower alcohols in the presence of acetone permitted the generation of crystals of 0.001 to 0.005 cubic mm with varying shapes.

From the above mentioned analytical data it is evident that such methods are not suitable for production of GH-crystals in larger scale. Furthermore, the growth time of the crystals used for X-ray analysis are of a duration from several weeks to one year, i.e. these attempts to crystallize GH cannot be used for production of GH-crystals in large quantities. Seen from an industrial point of view the references give no solution to the above mentioned problems.

From the literature it is well known that the presence of divalent cations during the process of crystallization permits not only excellent orientation during analysis, but also improved physical conditions for the crystallization of insulin (e.g. US pat. no. 2174862).

Growth hormone is more than three times larger than insulin and has a totally different conformation. Surprisingly the addition of cations to solutions containing B-hGH have now permitted the generation of stable, uniform crystals of the growth hormone in high yields. Furthermore, the time used for the formation of high quality B-hGH crystals is relatively short.

The general use of cations to form salts with biologically active polypeptides in solutions are known. This approach has been used in pharmaceutical preparations to give prolonged release of the polypeptides when used for parenteral administration. See: Mikaelson, 1983, Blood 62, 1006, and Wang, Y-C. J. and Hanson, M.A. (1988) Journal of Parenteral Sci. and Tech. 42, 3-26.

Bovine growth hormone has been formulated for veterinarian use in a mixture of divalent ions and an oil (EP 343 696). By addition of $ZnCl_2$ to either bovine or ovine growth hormone in the presence of lipids undefined particles were produced to form a prolonged release formulation. The growth hormone was dispersed in the carrier in such a way as to trap 1 to 4 Zn molecules per growth hormone molecule. The solutions were prepared in the presence of varying concentrations of denaturing solutes (1 to 4 M of urea) at high pH (9.5). It is not likely that the mentioned particles should be crystals and the patent application does not state so.

Summary of the invention

The present invention relates to crystallization of human growth hormone (hGH), human Met-growth hormone, truncated forms of human growth hormone and derivatives of these, i.e. specimens of the above mentioned proteins where one or a few amino acids have been exchanged for other amino acids.

The invention also relates to crystallization of growth hormones (GH) and forms hereof as mentioned above from other

species than homo sapiens, e.g. bovine, porcine and ovine growth hormone.

The present invention primarily relates to the generation of chemically stable and bioactive cation-GH crystals.

The invention also relates to a well defined process for production of both larger and smaller crystals of growth hormone.

The invention is further characterized by the usefull application of the said crystals in the treatment of GH disorders and for other medical purposes.

The invention is characterized by the formation of large uniform crystals of GH by supplementing the solutions with cations. An example of such cationic supplementation is Zn^{++} but also other cations of either inorganic (Na, Ca or other cations) or of organic nature (protamine, polyArg, polyLys or mixtures thereof) can be used advantageously. Even mixtures of these cations e.g. Zn^{++} and protamine can be used. The above mentioned polyArg and polyLys may have a chain length of 5 to 100 amino acids.

The invention is further characterized by the selective use of well defined buffers for the formation of crystals of GH or derivatives thereof, among others the formation of small uniform crystals in dilute phosphate or acetate buffer at pH 5.5 to 8.0.

In a preferred embodiment the invention is also characterized by the addition of organic solvents to the buffers. A variety of short chain aliphatic or cyclic alcohols and/or ketones can be used in the process of generation of crystals of GH or derivatives thereof. Acetone in concentrations of 0.1 to 20% can be used and preferably 10% is used for the formation of smaller sized crystals. Furthermore, supplementation of the

buffers with ethanol (1 to 50%, preferably 5 to 10%) also promotes crystal formation.

The invention is further characterized by the formation of crystals in large volumes of buffer solutions, thus providing a valuable tool for fast and efficient down stream processing of GH or derivatives thereof.

The invention provides for the controlled formation of crystals in different categories with respect to size. The size distribution is strongly dependent on the Zn^{++} to GH ratio. Micro crystals are preferably formed at a Zn^{++} to GH ratio of 2 to 5 mol to mol with a yield of 50 to 95%. Larger crystals (8 to 50 μm) are formed when the Zn^{++} to growth hormone ratio is ajusted between 0.5 to 2.0 mol to mol.

Objects and advantages

The invention provides a method apt for a pharmaceutical preparation with high chemical stability compared with the current hGH preparations based upon a lyophilized amorphous product.

The invention also provides a valuable tool for the developments of prolonged release pharmaceutical preparations.

Furthermore, the invention provides a valuable tool for production and purification purposes of GH.

Detailed description of the invention

Specifically the invention applies a set of unique conditions that as a whole concept implies the high quality and yield of the said crystals.

1. The starting material, the growth hormone that may be of any origin and if desired derivatized, is applied in a concentration greater than 0.1 mg/ml, preferably 4 to 7 mg/ml and most preferred about 6 mg/ml.

The conductivity of the buffer systems e.g. phosphate buffer (NaH_2PO_4) or acetate buffer ($NaAc/NH_4Ac$) may be varied between 0.2 mS/cm to 9.0 mS/cm, corresponding to a concentration of the buffer components within the range of 5 to 50 mM, preferably 5 to 20 mM and most preferred 9 to 11 mM.

The pH may be varied between 5,5 to 8,0 preferably between 5,8 to 7,5.

2. To the above mentioned solution may be added an organic solvent.

Acetone is used in a concentration which may vary between 0,1 to 20%, preferably 5 to 15%, and most preferred 9 to 11%.

Other solvents e.g. ethanol, methanol, propanol or phenols may be used alone or as a mixture instead of or together with acetone in a concentration within the range of from 1 to 50%, except for phenols where the range is between 0.1 to 5%.

3. To the resulting solution is added cations of inorganic or organic nature, or mixtures thereof.

Zn^{++} is preferably used in a concentration from 0.5 mol/mol GH to 5 mol/mol GH.

By addition of cations of inorganic nature other than Zn^{++} the concentration may be varied between 0.5 mol/mol GH and 10 mol/mol GH.

The concentration of cations of organic nature (protamine; polyArg a.s.o.) may be varied between 0.02 mol/mol GH and 10 mol/mol GH.

The concentration of mixtures of cations (Zn^{++} and protamine a.s.o.) may be varied between 0.02 mol/mol GH and 10 mol/mol GH.

4. The crystals are grown for a period of from 1 to 120 hrs. preferably 5-72 hrs., most preferred 20-48 hrs., and at a temperature of from 4 to 30°C.

5. In the further processing the crystals are recovered by centrifugation or filtration, followed by washing and/or freeze drying to remove remaining organic solvents. Thereupon the crystals are analyzed to test the purity for acceptance for pharmaceutical use.

Preparations can now be formulated by adding various selected buffers and other pharmaceutical additives to ensure that the activity of GH is maintained and undesirable reactions during processing and storage are minimized.

The invention is further illustrated but not limited by the following examples:

Example 1

Crystallization of hGH in the presence of Zn^{++}.

500 ml of hGH solution in a concentration of 6 mg/ml was incubated in 10 mM phosphat buffer (NaH_2PO_4) and adjusted to pH 6.1 with H_3PO_4. Acetone was added to a final concentration of 10% (v/v) and thereafter zinc acetate solution was added to a final concentration of 0.08 mg $ZnAc_2$, $2H_2O$/ml.

The resulting solution was left at 15°C for 20 hours, whereby crystals were allowed to form.

After this the crystals were recovered and washed 3 times with crystallization buffer without acetone. The crystallization was checked by microscopy and the size of the crystals were measured to 8-12μm. A photomicrograph is shown in Figure 1.

The crystal yield of hGH was determined by solubilization of the washed crystals in 7M urea followed by ion exchange HPLC analysis.

The yield was found to be more than 50%.

Example 2

Example 1 was repeated with the exception that the addition of acetone was omitted.

The crystals of hGH resulting from this procedure were much smaller than the crystals resulting from Example 1, less than 2 μm.

Example 3

Formulation of an injection preparation containing hGH

Crystals produced as described in example 1 was filtrated and washed in pure sterile water. Then the crystals were placed in a freeze dryer and vacuum dried for 1½ hrs. at 20-23°C to remove remaining organic solvents. An analysis showed that the isolated crystals exhibited a purity which was acceptable for pharmaceutical purposes.

A pharmaceutical injection preparation was prepared at pH 6.4 according to the following formulation:

Ingredient	Concentration
HGH crystals	4 IU/ml
$NaH_2PO_4, 2H_2O$	2.8 mg/ml
Glycerol	14.0 mg/ml
Benzyl alcohol	15.0 mg/ml
$Zn(Ac)_2, H_2O$	0.08 mg/ml

Example 4

Example 3 was repeated with the exception that $Zn(Ac)_2$, H_2O was omitted, giving the following preparation:

Ingredient	Concentration
HGH crystals	4 IU/ml
NaH_2PO_4, $2H_2O$	2.8 mg/ml
Glycerol	14.0 mg/ml
Benzyl alcohol	15.0 mg/ml

Example 5

The crystals were treated in the same way as in example 3, and the following formulation was used at pH 6.1:

Ingredient	Concentration
HGH crystals	4 IU/ml
NaH_2PO_4, $2H_2O$	2.14 mg/ml
NaCl	9.0 mg/ml

Example 6

Tibia test

To estimate the in vivo biological potency of the hGH crystals prepared according to the invention a tibia test was performed using hypophysectomized rats. The test was performed in accordance with the method described in the European Pharmacopoeia.

Two preparations of hGH crystals F-7 and F-8 each containing an estimated amount equivalent to 4 IU were tested against a dissolved standard hGH preparation.

The following results were obatined:

Table 1

The potency of the preparations F-7 and F-8

Test preparat.	Potency % of std.	IU/vial	95% confid. limits, % of std.
F-7	90.1	3.9	87.6 - 114.1
F-8	103.8	4.5	90.6 - 110.4
Std. B-hGH 1986	≡ 100.0	≡ 4.0	-

From the performed test it can be concluded that the hGH crystals according to the invention are equally biological potent as the solubilized hGH standard and therefore must have an equal bioavailability compared with usual solubilized hGH.

CLAIMS

1. A process for production of bioactive cation-GH crystals or crystals of derivatives of GH, comprising the following steps:

a A solution of GH or derivatives thereof in a concentration greater than 0.1 mg/ml is incubated in a buffer system with a conductivity between 0.2 to 9.0 mS/cm, corresponding to a concentration of the buffer components within the range of between 5 and 50 mM, at a pH between 5.5-8.0.

b To the above mentioned solution is added an organic solvent or a mixture of solvents in a concentration from 0.1 to 50%.

c Further is added cations of inorganic or organic nature or mixtures thereof in a concentration from 0.02 mol/mol GH to 10 mol/mol GH.

d Crystals are grown at a temperature from 4 to 30°C.

2. A process according to claim 1 wherein the growth hormone is hGH or derivatives thereof.

3. A process according to claims 1-2 wherein the buffer system contains phosphate buffer or acetate buffer.

4. A process according to claims 1-3 wherein the organic solvent is acetone in a concentration of 0.1 to 20%.

5. A process according to claims 1-3 wherein step b, addition of organic solvent, is omitted.

6. A process according to claims 1-5 wherein the cation is Zn^{++} in a concentration from 0.5 mol/mol GH to 5 mol/mol GH.

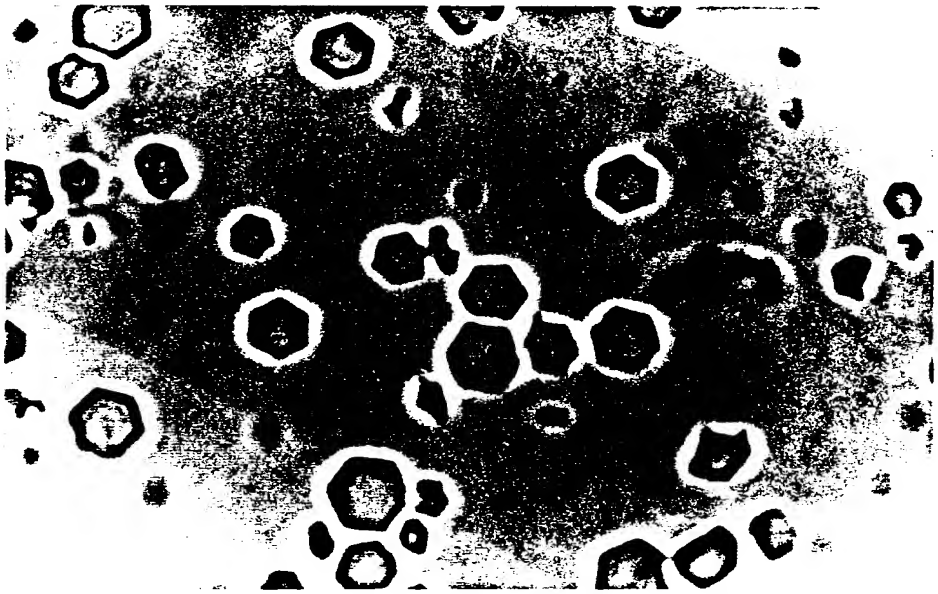

Fig. 1

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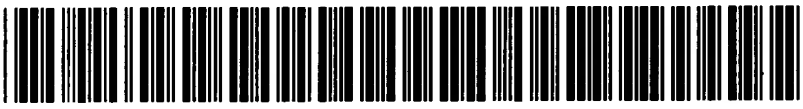

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